Expressional changes in stemness markers post electrochemotherapy in pancreatic cancer cells

Expressional changes in stemness markers post electrochemotherapy in pancreatic cancer cells.

M Shahzad Ali[1,2], Giuseppe Saglio[2], Daniela Cilloni[2], KS Gill[1], Declan M Soden[1], Patrick F Forde[1,3]

[1]Cork Cancer Research Centre, Western Gateway Building, University College Cork. Ireland.

[2]Department. of Clinical and Biological Sciences, University of Turin, San Luigi Hospital, Regione Gonzole 10, 10043, Turin, Italy.

[3]Corresponding author:

Dr. Patrick F Forde

Cork Cancer Research Centre

Western Gateway Building

University College Cork.

Ireland.

Tele: +353 (0)21 4205706

Email: p.forde@ucc.ie

Abstract

Pancreatic cancer is one of the most lethal cancers with high metastatic potential and strong chemoresistance. The capability of a tumor to grow and propagate is dependent on a small subset of cells within a tumor, termed cancer stem cells. Cancer stem cells exhibit great tumorigenicity and are closely correlated with drug resistance and tumor recurrence. The aim of our study was to illustrate electrochemotherapy as an effective treatment for pancreatic cancer along with the expression change in stemness genes (Nanog, Sox2 and Oct3/4) in pancreatic cancer cells post electrochemotherapy with bleomycin, cisplatin and oxaliplatin. Our results showed the enhanced expression of Nanog and decreased expression level of Oct3/4 after electrochemotherpy. We thus propose that these stemness marker may have important roles in the initiation and/or recurrence of pancreatic cancer, and consequently may serve as important molecular diagnostics and/or therapeutic targets for the development of novel treatment strategies in pancreatic cancer patients. In conclusion, targeting these stemness factors could potentially improve electrochemotherapy as a treatment and preventing recurrence.

Keywords: Pancreatic; stemness; recurrence; electrochemotherapy

1. Introduction

Pancreatic cancer continues to be one of the deadliest cancers with a high metastatic potential and strong chemoresistance [1]. The incidence of pancreatic cancer increases each year with no significant decrease in mortality [2]. Almost 90% of pancreatic malignancies are pancreatic ductal adenocarcinomas (PDACs) [3]. PDAC is associated with a poor prognosis and has become the fourth most common cause of cancer-related mortality. The 5-year survival of patients is dismal at 6% or less [3].

Currently for early disease stage surgery followed by adjuvant therapy is the treatment option for most patients. However, majority of patients present with locally advanced disease or metastatic disease which makes resection difficult [4]. Conventional therapeutic strategies have resulted in decreased perioperative morbidity and mortality after pancreatic resection, but this has not improved the median overall survival of patients with localized operable pancreatic cancer. Thus, there is a need for alternative therapeutic strategy is needed to improve the therapeutic situation of patients with PDAC [4].

Electrochemotherapy (ECT) is a cancer treatment, a non-thermal cell permeabilisation technology that renders the treated cell membranes permeable to otherwise impermenant or poorly permeant chemotherapeutics [5]. The use of ECT for tumor treatments leads to a local potentiation of chemotherapy by reducing the doses of the drugs, minimizing the side effects, and increasing the efficacy of chemotherapy [6]. ECT is regularly used for cutaneous and subcutaneous metastases and is an effective treatment and with the advancement of new medical devices this is now a treatment option for deep seated tumors including pancreatic cancer [7]. One concerning feature of pancreatic cancer is the high resistance to treatments. Even for patients with resectable disease, most of them will relapse [8]. Therefore, it is crucial to understand mechanisms that cause treatment resistance and is crucial to help develop new effective treatments for this deadly disease. PDAC has a high level of Cancer Stem Cells (CSCs) [9]. CSCs are highly resistant to conventional chemotherapy and radiotherapy and are considered a cause of tumor relapse after eradication of the tumor bulk [10]. Stemness factors Nanog and Sox2 are highly expressed in human embryonic stem (ES) cells with other stem cell marker, Lin28 and Oct4 together can reprogramed human somatic cells to pluripotencey [11]. The stemness factor, Nanog, has been shown to be upregulated in colorectal, hepatic, and brain cancer stem cells [12,13]. In brain tumors the CSCs were reported to express various stemness specific proteins such Sox2 and Oct3/4 [14]. Oct4/Sox2 heterodimers control the expression of Nanog, in which the heterodimers bind to the octamer/sox elements within the Nanog proximal promoter region and stimulate the Nanog transcription [15,16]. The upregulated expression of Nanog, Sox2 and Oct3/4 stemness genes have being involved in tumorigenicity, tumor transformation, tumor metastasis [17], and recurrence after chemoradiotherapy [18].

In the present study we determine whether ECT can modulate stemness inducing proteins (Oct4/3, Nanog, and Sox2) and measure the differential expression of these proteins in PDAC cells. These proteins may be the key players in the development of resistance, recurrence and disease progression after electrochemotherapy.

2. Methods:

2.1. Cell culture

The established human pancreatic cancer cell line PANC-1 was obtained from the *American Type Culture Collection* and the murine pancreatic cell line, Pan02 was obtained from the National Cancer Institute at Frederick. Both cell lines are well established models for the study of pancreatic cancer [29,30]. PANC-1 cells were maintained in Dulbeccos Modified Media supplemented with 20% (v/v) fetal calf serum, whilst Pan02 cells were maintained in RPMI 1640 medium with 10% (v/v) fetal calf serum. Both cell lines were supplemented with 1% penicillin/streptomycin, and were grown at 37°C, 5% CO_2.

2.2. Drug concentrations

PANC-1: Bleomycin: 0.1µg/ml; 0.5µg/ml; 1µg/ml and 1.5µg/ml, Cisplatin: 0.1µg/ml; 0.2µg/ml; 0.5µg/ml and 1.5µg/ml, Oxaliplatin: 1µg/ml, 2µg/ml, 3µg/ml and 4µg/ml

Pan02: Bleomycin: 0.1µg/ml; 1µg/ml and 2.5µg/ml, Cisplatin: 0.5µg/ml; 1µg/ml; 2.5µg/ml and 5µg/ml, Oxaliplatin: 0.2µg/ml; 0. 5µg/ml; 1µg/ml and 2µg/ml.

2.3. Reversible electroporation and drug dose optimization

Clonogenic assays were performed to determine the cells recovery after reversible electroporation with Bleomycin, Cisplatin or Oxaloplatin. Following harvesting, $2x10^6$ cells were resuspended in 800µl of HEPES electroporation buffer (10 mM HEPES (Lonza), 250 mM sucrose and 1 mM $MgCl_2$ in sterile water). Half of the cells suspension were electroporated in 4mm cuvettes (VWR) in the presence of a range of drug concentrations (mentioned in section 2.2) of three different chemotherapeutic drugs bleomycin, cisplatin and oxaliplatin separately and other half of the suspension ($1x10^6$/400 µl) was used as passively treated cells. Previously optimized parameters for reversible electroporation used were: 8 pulses of 99µs at a frequency of 1Hz with 0.6kv/cm for PANC-1 cells or 1kv/cm for Pan02 cells using a BTX electroporator.

Both actively (ECT) and passively treated cells with respective drugs along with untreated cells (control) were rested in the incubator at 37 ºC for 30 minutes. After that, cells from all groups (actively and passively treated and untreated) were seeded in 6-well plates at densities as follows: 1500 cells per well for PANC-1 and 500 cells per well for Pan02, and allowed to grow for ten days. To visualize colonies, media was removed, cells were fixed in 96% ethanol for 10 min and stained with Prodiff solution C (Braidwood laboratories 22009). Plates were scanned using the Odyssey IR imaging system (Li-Cor, Cambridge, United Kingdom) and colonies quantified. Results are presented as integrated intensity ± SEM from at least three independent experiments.

2.4. Cell viability through PI uptake

Propidium iodide (PI) is a small fluorescent molecule that binds to DNA but cannot passively traverse into cells that possess an intact plasma membrane. PI uptake versus exclusion can thus be used to discriminate dead cells, in which plasma membranes become permeable regardless of the mechanism of death, from live cells with intact membranes. After electroporation cells were seeded at a density of 32000 cell/ml and 16000 cell/ml (PANC-1) and 16000 cells/ml and 8000

cells/ml (Pan02) cells in a total of 2 ml in 6 well plates, for 24, 48hrs respectively. After the given time, cells were typsinised, washed in PBS and then propidium iodide added at 5ug/100ul. Samples were then run on the BD LSR II instrument.

2.5. Evaluation of cell morphology

Morphological features of cells treated by electroporation with bleomycin, cisplatin, and oxalipaltin were examined by light microscopy to confirm successful electroporation. Aliquots of untreated control and drug-treated were cytospun onto glass slides and stained using Pro-Diff (Braidwood Laboratories BAPROD1–fixed and stained with buffered eosin followed by methyl thionins). Cytospin images are representative of at least three independent experiments.

2.6. Colony formation assay

The ability of cells to recover from treatments and form colonies as a monolayer was assessed using a colony formation assay. Following treatment, either 250cells/ml of PANC-1 or 100cells/ml of Pan02 cells were reseeded into a well of a six-well plate (in triplicate) each well contained of 4ml of nutrient medium. Cells were allowed to adhere and grow for one to two weeks. Visualization was carried out as per method in Section 2.3.

2.7. Immunofluorescence

Both cell lines were grown on cover slips for 48hours and then fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.1% Triton X100. After blocking with 10% Fetal Bovine Serum + 5% BSA + 1% fish gelatine in PBS 1x for 1hr, cells were washed 3 times with 1XPBS and then incubated for 2 hours at room temperature or overnight at 4^{o}C with the primary antibody of interest diluted 1:250 in blocking solution [antibodies used: monoclonal Sox-2 Ab (E-4): sc-365823, monoclonal Nanog (H-2): sc-374103 and the monoclonal Oct4 (C-10): sc-5279] (Santa Cruz Biotechnology, Heidelberg, Germany). and then washed 3 times with PBS for 5 minutes. The antigen-antibody complex was detected by incubation for 45 min with appropriate secondary antibody. The secondary antibodies Alexa Fluor 488 labeled with fluorescein (FITC) goat anti-mouse IgG (Invitrogen Life Technologies, Carlsbad, CA) was used in a dilution of 1:500. The cells were then washed in PBS for 5 minutes and the nuclei were stained with a solution of DAPI. Cells were analyzed on a fluorescence microscope (Leica DM2000 LED, Leica microsystem, Germany) and images acquired using Leica application Suite 4.4.0 software. For each sample, repeated in triplicate, were performed three independent measurements and each measurement was normalized on background noise.

2.8. Western blotting

Following 48hours post treatment total cellular protein extracts were prepared by scraping the cells with modified RIPA buffer (50 mM Tris HCl (pH 7.4), 150 mM NaCl, 0.25% sodium deoxycholate, 1% Igepal, 1 mM EDTA, 1× Pefabloc, 1× protease inhibitor cocktail, 1 mM Na_3VO_4, 1 mM NaF). Equal concentrations (70ug) of each protein samples were separated on NuPAGE 4–12%, Bis-Tris gels (Invitrogen, NP0322) and electrophoretically transferred onto PVDF membranes. The membrane was incubated overnight at 4 degrees with the primary antibody of interest diluted 1:250 in TBS-1X-tween 0.1% solution [antibodies used: monoclonal Sox-2 Ab (E-4): sc-365823, monoclonal Nanog (H-2): sc-374103 and the monoclonal Oct4 (C-10): sc-5279] (Santa Cruz Biotechnology, Heidelberg, Germany). Protein expression was visualized using the Odyssey IR imaging system (Li-Cor goat anti-mouse IgG 926 32211 IRDy

800). Level of expressional change was assessed with band intensity in comparison to untreated control.

3. Results:

3.1. Reversible electroporation and drug dose optimization

Reversible electroporation is the application of an electrical field to increase the permeability of cell membranes to drugs that are otherwise impermeable. ECT potentiates the efficacy of drugs in tumors, whilst allowing for a lower therapeutic dose and subsequently reducing side effects. It was observed that ECT significantly increased the efficacy of chemotherapies in both cell lines with optimized EP parameters mentioned in section 2.3. **Figure 1a and 1b** presents the recovery of PANC-1 and pan02 cell lines post treatment. The cell lines were exposed to a range of drug concentrations either by active (ECT) or passive treatment. The optimized dose for each drug was identified by the recovery of cells post treatment. Passive treatment of each dose was compared to ECT with the same dose. For all drugs ECT decreased the ability of cells to recover and proliferate. We selected those drug concentrations for all drugs as an optimized dose which has relatively less/no effect on cell recovery in passive treatment compared to dramatic reduction of cell recovery against ECT with same dose. The optimized drug concentration was identified as 0.1µg/ml for Bleomycin in both cell lines, 0.2µg/ml of Cisplatin in PANC-1 and 3µg/ml in Pan02, and 3µg/ml of Oxaliplatin in PANC-1 and 0.2µg/ml in Pan02 as shown by blue dotted rectangles in **Figure 1a and 1b**. The recovery assay revealed the cells treated with ECT-Bleomycin recovered less than the cells treated with ECT-Cisplatin and ECT-Oxaloplatin. This data suggests that bleomycin was a more potent chemotherapy drug as compared to cisplatin and oxaliplatin.

3.2. Morphology and viability of pancreatic cells

Treatment regimens of the pancreatic cells (PANC-1 and Pan02) included electroporation only, chemotherapy only, electrochemotherapy and untreated cells. 24hrs post treatment aliquots were removed for viability assessment. Morphological analysis revealed electroporation only induced transient changes including the presence of cytoplasmic vacuoles which confirmed that reversible electroporation had been taken place as indicated by green arrows in **Figure 2b (ii)** and **Figure 3b (ii)**. Electroporation in the absence of any chemotherapy has changed the morphological characteristics of both cells types revealing the change in membrane potential and higher cell death as compared with untreated cells as shown in **Figure 2 (a-i, b-ii)** and **Figure 3(a-i, b-ii)**. Electroporation leads to increased uptake of chemotherapeutic agents during electrochemotherapy. Passive treatment showed the cytotoxic effect but the cell death increased approx. 2 times with the electrochemotherapy with each drug. Red arrows indicate dead cells (having no cytoplasmic contents) and blue arrows indicating dying cells (having unstable cytoplasmic content and disintegrated genomic contents) shown in **Figure 2** and **Figure 3**. Morphological changes also revealed that the size of cells after electrochemotherapy were increased in comparison to passive treatment with bleomycin, cisplatin and oxaloplatin as shown in **Figure 2b (iv, vi, vii)** and **Figure 3b (iv, vi, vii)**. Enlargement of cells and especially the disintegration of nuclear content was more obvious in case of bleomycin and oxaliplatin. Bleomycin is more potent as compared to cisplatin and oxaliplatin because it caused more cell

death as shown in **Figures 2b (iv)** and **Figure 3b (iv)**. This is also a strong indication of both the reversible mode of electroporation and the intracellular modification that with the passage of time cells modified themselves to efflux the drug but bleomycin molecule is as much larger as the cell after intracellular modifications were not able to get rid of bleomycin, which was the source of more cell death due to cytotoxicity which can be predicted from **Figure 2** and **Figure 3.**

3.3. Recovery of pancreatic cells post treatment

Colony formation ability of both cell lines were comparatively higher in electroporation only and when the cells were passively treated for 30 minutes with bleomycin, cisplatin and oxaloplatin which could be predicted from **Figure 4** and **Figure 5**. Whist the pattern of cell recovery was dramatically decreased in both cell lines when they were electroporated in the presence of these drugs. The ability to recover in case of ECT-Bleomycin was very low in both PANC-1 and Pan02 in comparison to cisplatin and oxaloplatin, as shown in **Figure 4 and Figure 5** respectively. The recovery of cell in any case of drug treatment indicate there are some mechanisms/intracellular modification has been taken place in cancer cells which helped them to detoxify the effect of such a strong electrochemical treatment to reoccur again after the passage of time. One of possible modification might be the expressional changes in pluripotency factor which were the source to provide cancer cells the stem cell properties.

3.4. Stemness factors expression in pancreatic cells post electrochemotherapy

We have observed that both cell lines express Nanog, Sox2 and Oct4. As shown in **Figure 6a** and **7a**, the expression of Oct3/4 decreased in drug treated cells as compared to untreated cells for both cell lines. Nanog expression was increased in the cells after electrochemotherapy in both cell lines shown in **Figure 6c and 7c**, whilst the expression of stem cell marker Sox2 remained constant in as shown in **Figure 6b and 7b**. To con firm the validity of expression pattern of stem cell marker as shown in immunofluorescence, western blot was performed. At the protein level, the expression patterns of these stem cell markers Nanog, Sox2 and Oct4 were parallel those of their respective immunofluorescence, as shown in the **Figure 8**. The red dotted box in **Figure 8(i, a)** indicates the higher band intensity for enhanced expression of Nanog after electrochemtherapy in both cell lines as compared to untreated control and passively treated samples, and similarly the blue dotted box in **Figure 8(ii, b)** showed the decreased in band intensity for Oct3/4 in treated cell samples which indicate downregulation of Oct3/4 in drug treated samples in comparison to untreated control.

4. Discussion/Conclusion:

For many chemotherapeutic agents the response rate is low because the chemotherapy cannot readily reach their intracellular site of action. For such drugs, the cell membrane is often a significant barrier that reduces effectiveness by restricting intracellular access. Electroporation uses short intense electric pulses to increase the plasma membrane permeability which allows the introduction of non- or poorly permeant molecules into the cells [5-6]. Electrochemotherapy (ECT) is the combination of chemotherapeutic drug with these optimized pulses which augment the drug delivery inside the cell and therefore increasing the efficacy of chemotherapy.

Electrochemotherapy has increased the effectiveness of non-permeant chemotherapeutic drugs such as bleomycin by several fold. Overall the effectiveness of electrochemotherapy is approximately 80% and is considered an effective and safe local ablative treatment [19]. Studies have indicated other mechanisms including vascular disrupting effect and immune response are also involved during the application of electrochemotherapy which help to make this treatment application very attractive in the clinic [7,20,28]. Electrochemotherapy has been established as an effective treatment of skin tumors of different histology and has been successful in the treatment of internal tumors such as liver and colorectal [21].

In the present study we examined the viability and recovery of pancreatic cancer cells post electrochemotherapy with bleomycin, cisplatin and oxaliplatin. Electrochemotherapy with bleomycin exhibited a higher cell death rate over cisplatin and oxaliplatin. Morphological changes revealed that the size of cells after electrochemotherapy were increased as compared with passive treatment with bleomycin, cisplatin and oxaliplatin. Enlargement of cells and especially the disintegration of nuclear content was more obvious in case of bleomycin indicating its potent cytotoxicity and therefore demonstrating ECT as a very effective treatment. Recovery of the pancreatic cancer cells were also measured which showed that the cells recovered to some degree after electrochemotherapy. The ability to recover in case of electrochemotherapy with bleomycin was low in both cell lines in comparison to cisplatin and oxaliplatin. This recovery represents a major challenge to this treatment and if unresolved may lead to recurrence.

The recovery of cells in any drug treatment indicates there are some mechanisms or intracellular modifications that has been taken place in the treated cells which helped them to detoxify the effect of drugs. One possible modification might be the expression changes of stemness genes that has the potential to generate recurrence in pancreatic cancer cells after electrochemotherapy and such expressional changes in these genes give them the properties of cancer stem cells CSC, a small and distinct population of cancer cells that mediates tumoregenesis, metastasis and resistance to standard treatments. Evidence for the existence of cancer stem cells was first reported in human acute myeloid leukemia in the 1990s and subsequently were identified in various solid tumors including breast cancer, brain tumor, prostate cancer, pancreatic cancer, ovarian cancer [22]. The embryonic stemness inducing factor, Nanog sex determine region Y-

box 2 (Sox2), octamer-binding transcription factor 4, Oct4, for instance, were shown to be highly express in the CSCs in hepatic, colorectal, and brain CSCs [12,13,14]. The Oct4/Sox2 heterodimers, regulate the expression of Nanog by binding to the octamer/sox elements within the Nanog proximal promoter region and induces Nanog transcription [15,16]. The critical role of these factors in reprogramming processes makes them essential not only for embryonic development but also tumorigenesis. Recent data showed that the overexpression of Nanog, Sox2 and Oct3/4 together or separately, led to tumor transformation, tumorigenicity, tumor metastasis, and even distant recurrence after chemoradiotherapy [23]. Data from our study indicate that electrochemotherapy has an effect on the stemness-associated markers. Many anti-cancer therapies are evaluated based on their ability to shrink tumors [24], but if the therapies are not targeting the cancer stem cells, then the tumor will most likely relapse. The cancer stem cell theory suggests that a few cancer cells can act as stem cells and reproduce themselves causing recurrence [25].

Our study revealed that the expression of master stemness inducing factor Nanog was increased in the cells electroporated in the presence of drugs in both human and murine cell lines (**Figure 6c and 7c**), whilst the expression of Oct3/4 decreases with the treatment in both PANC-1 and Pan02 cell lines. Interestingly the expression of stem cell marker Sox2 remain relatively constant in both cell lines as shown in **Figure 6b and 7b**. As one approach to better understanding the functional significance of Sox2, we examined the expression in both the PANC-1 and Pan02 lines by immunofluorescence indicated that it mainly localized to the cell nucleus (**Figure 6a and 7b**) that indicate its role in the transcription of master stemness marker the Nanog [26, 27]. Whilst the decreased in expression of Oct4 correlated with the expression of Akt which promote the self-renewal and cell survival of cancer cells [26]. To confirm the validity of expression pattern of stem cell marker as shown in immunofluorescence, western blot was performed. At the protein level, the expression patterns of these stem cell markers Nanog, Sox2 and Oct4 were parallel to those of their respective immunofluorescence, as shown in the **Figure 8**.

In conclusion, we present data demonstrating changes in stemness factors after electrochmeotherapy. The altered expression of the stemness markers have the potential to allow for recurance post treatment. With the advancement of electrochemotherapy as a cancer treatment for deep seated tumors including pancreatic cancer our results warrant further investigation to improve upon the treatment.

References:

1. Rahib L, Fleshman JM, Matrisian LM, Berlin JD. Evaluation of Pancreatic Cancer Clinical Trials and Benchmarks for Clinically Meaningful Future Trials. *JAMA Oncol.* 2016;2(9):1209. doi:10.1001/jamaoncol.2016.0585.
2. Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting Cancer Incidence and Deaths to 2030: The Unexpected Burden of Thyroid, Liver, and Pancreas Cancers in the United States. *Cancer Res.* 2014;74(11):2913-2921. doi:10.1158/0008-5472.CAN-14-0155.
3. Feldmann G, Maitra A. Molecular genetics of pancreatic ductal adenocarcinomas and recent implications for translational efforts. *J Mol Diagn.* 2008;10(2):111-122. doi:10.2353/jmoldx.2008.070115.
4. Ansari D, Gustafsson A, Andersson R. Update on the management of pancreatic cancer: Surgery is not enough. *World J Gastroenterol.* 2015;21(11):3157-3165. doi:10.3748/wjg.v21.i11.3157.
5. Mir LM, Orlowski S, Belehradek J, Paoletti C. Electrochemotherapy potentiation of antitumour effect of bleomycin by local electric pulses. *Eur J Cancer.* 1991;27(1):68-72. http://www.ncbi.nlm.nih.gov/pubmed/1707289. Accessed February 17, 2018.
6. Sersa G, Miklavcic D, Cemazar M, Rudolf Z, Pucihar G, Snoj M. Electrochemotherapy in treatment of tumours. *Eur J Surg Oncol.* 2008;34(2):232-240. doi:10.1016/j.ejso.2007.05.016.
7. Miklavcic D, Snoj M, Zupanic A, Kos B., Cemazar M., Kropivnik M., Bracko M, Pecnik T., Gadzijev E. Sersa G. Towards treatment planning and treatment of deep-seated solid tumors by electrochemotherapy. *Biomed Eng Online.* 2010;9:10. doi:10.1186/1475-925X-9-10.
8. Chand S, O'Hayer K, Blanco FF, Winter JM, Brody JR. The Landscape of Pancreatic Cancer Therapeutic Resistance Mechanisms. *Int J Biol Sci*. 2016;12(3):273-282. doi:10.7150/ijbs.14951.
9. Durko L, Wlodarski W, Stasikowska-Kanicka O, Wagrowska-Danilewicz M, Danilewicz M., Hogendorf p, Strzelczyk J, Malecka-Panas E. Expression and Clinical Significance of Cancer Stem Cell Markers CD24, CD44, and CD133 in Pancreatic Ductal Adenocarcinoma and Chronic Pancreatitis. *Dis Markers*. 2017;2017:1-7. doi:10.1155/2017/3276806.
10. Mitra A, Mishra L, Li S. EMT, CTCs and CSCs in tumor relapse and drug-resistance. *Oncotarget*. 2015;6(13):10697-10711. doi:10.18632/oncotarget.4037.
11. Shan J, Shen J, Liu L, Xia F, Xu C, Duan G, Xu Y, Ma Q, Yang Z, Zhang Q, Ma L, Liu J, Xu S, Yan X, Bie P, Cui Y, Bian XW, Qian C.l. Nanog regulates self-renewal of cancer stem cells through the insulin-like growth factor pathway in human hepatocellular carcinoma. *Hepatology*. 2012;56(3):1004-1014. doi:10.1002/hep.25745.

12. Zhang J, Espinoza LA, Kinders RJ, Lawrence SM, Pfister TD, Zhou M, Veenstra TD, Thorgeirsson SS, Jessup JM.. NANOG modulates stemness in human colorectal cancer. *Oncogene*. 2013;32(37):4397-4405. doi:10.1038/onc.2012.461.
13. Zbinden M, Duquet A, Lorente-Trigos A, Ngwabyt S-N, Borges I, Ruiz i Altaba A. NANOG regulates glioma stem cells and is essential in vivo acting in a cross-functional network with GLI1 and p53. *EMBO J*. 2010;29(15):2659-2674. doi:10.1038/emboj.2010.137.
14. Hemmati HD, Nakano I, Lazareff JA, Masterman-Smith M, Geschwind DH, Bronner-Fraser M, Kornblum HI.Cancerous stem cells can arise from pediatric brain tumors. *Proc Natl Acad Sci*. 2003;100(25):15178-15183. doi:10.1073/pnas.2036535100.
15. Kuroda T, Tada M, Kubota H, Kimura H, Hatano SY, Suemori H, Nakatsuji N, Tada T. Octamer and Sox Elements Are Required for Transcriptional cis Regulation of Nanog Gene Expression. *Mol Cell Biol*. 2005;25(6):2475-2485. doi:10.1128/MCB.25.6.2475-2485.2005.
16. Do H-J, Lee W-Y, Lim H-Y, Oh J-H, Kim D-K, Kim J-H, Kim T, Kim J-H. Two potent transactivation domains in the C-terminal region of human NANOG mediate transcriptional activation in human embryonic carcinoma cells. *J Cell Biochem*. 2009;106(6):1079-1089. doi:10.1002/jcb.22089.
17. Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A, Weinberg RA. An embryonic stem cell–like gene expression signature in poorly differentiated aggressive human tumors. *Nat Genet*. 2008;40(5):499-507. doi:10.1038/ng.127.
18. Feske S. Calcium signalling in lymphocyte activation and disease. *Nat Rev Immunol*. 2007;7(9):690-702. doi:10.1038/nri2152.
19. Sersa G, Miklavcic D. Electrochemotherapy of tumours. *J Vis Exp*. 2008;(22). doi:10.3791/1038.
20. Mir LM. Bases and rationale of the electrochemotherapy. *Eur J Cancer Suppl*. 2006;4(11):38-44. doi:10.1016/J.EJCSUP.2006.08.005.
21. Coletti L, Battaglia V, De Simone P, Turturici L, Bartolozzi C, Filipponi F. Safety and feasibility of electrochemotherapy in patients with unresectable colorectal liver metastases: A pilot study. *Int J Surg*. 2017;44:26-32. doi:10.1016/j.ijsu.2017.06.033.
22. Kakarala M, Wicha MS. Implications of the Cancer Stem-Cell Hypothesis for Breast Cancer Prevention and Therapy. *J Clin Oncol*. 2008;26(17):2813-2820. doi:10.1200/JCO.2008.16.3931.
23. Ben-Porath I, Thomson MW, Carey VJ, et al. An embryonic stem cell–like gene expression signature in poorly differentiated aggressive human tumors. *Nat Genet*. 2008;40(5):499-507. doi:10.1038/ng.127.
24. Li, J., Sun, Z., Zhang, J., Shao, H., Miranda Cona, M., Wang, H., Marysael, T., Chen, F., Prinsen, K., Zhou, L., Huang, D., Nuyts, J., Yu, J., Meng, B., Bormans, G., Fang, Z., de Witte, P., Li, Y., Verbruggen, A., Wang, X., Mortelmans, L., Xu, K., Marchal, G. and Ni, Y.et al. A Dual-targeting Anticancer Approach: Soil and Seed Principle. *Radiology*. 2011;260(3):799-807. doi:10.1148/radiol.11102120.
25. Peitzsch C, Tyutyunnykova A, Pantel K, Dubrovska A. Cancer stem cells: The root of tumor recurrence and metastases. *Semin Cancer Biol*. 2017;44:10-24. doi:10.1016/j.semcancer.2017.02.011.
26. Lin, Y., Yang, Y., Li, W., Chen, Q., Li, J., Pan, X., Zhou, L., Liu, C., Chen, C., He, J., Cao, H., Yao, H., Zheng, L., Xu, X., Xia, Z., Ren, J., Xiao, L., Li, L., Shen, B., Zhou, H.

and Wang, Y.-J. Reciprocal Regulation of Akt and Oct4 Promotes the Self-Renewal and Survival of Embryonal Carcinoma Cells. *Mol Cell*. 2012;48(4):627-640. doi:10.1016/j.molcel.2012.08.030.
27. LING G-Q, CHEN D-B, WANG B-Q, ZHANG L-S. Expression of the pluripotency markers Oct3/4, Nanog and Sox2 in human breast cancer cell lines. *Oncol Lett*. 2012;4(6):1264-1268. doi:10.3892/ol.2012.916.
28. Ursic, K., Kos, S., Kamensek, U., Cemazar, M., Scancar, J., Bucek, S., Kranjc, S., Staresinic, B. and Sersa, G. Comparable effectiveness and immunomodulatory actions of oxaliplatin and cisplatin in electrochemotherapy of murine melanoma. *Bioelectrochemistry*. 2018;119:161-171. doi:10.1016/j.bioelechem.2017.09.009.
29. Suklabaidya S, Dash P, Das B, Suresh V, Sasmal PK, Senapati S. Experimental models of pancreatic cancer desmoplasia. *Lab Investig*. 2018;98(1):27-40. doi:10.1038/labinvest.2017.127.
30. Yan Y, Rubinchik S, Watson PM, et al. Establishing a Murine Pancreatic Cancer CaSm Model: Up-regulation of CaSm Is Required for the Transformed Phenotype of Murine Pancreatic Adenocarcinoma. *Mol Ther*. 2005;11(3):363-372. doi:10.1016/J.YMTHE.2004.09.023.

Figure legends:

Figure 1a. Chemotherapy dose optimization for Electrochemotherapy in PANC-1 cells. ECT dramatically reduced the capability of cancer cells to recover and proliferate relative to chemotherapy alone. Blue dotted rectangles indicate the optimized drug dose of bleomycin, cisplatin and oxaloplatin. Each well shown is a representative image of at least three independent experiments. Data also presented in line graphs as mean IKK ± SD M of three independent experiments.

Figure 1b. Chemotherapy dose optimization for Electrochemotherapy in Pan02 cells. ECT dramatically reduced the capability of cancer cells to recover and proliferate relative to chemotherapy alone. Blue dotted rectangles indicate the optimized drug dose of bleomycin, cisplatin and oxaloplatin. Each well shown is a representative image of at least three independent experiments. Data also presented in line graphs as mean IKK ± SD M of three independent experiments.

Figure 2: Morphological changes viability after electroporation in PANC-1. Following 24hours post treatment viability was analyzed through flow cytometry and morphology by cytospinning. Electroporation leads to transient morphological changes in pancreatic cancer cells including the presence of cytoplasmic vacuoles and leads to increased uptake of drugs with correspondence to their viability. Red arrows indicate the dead cells and blue arrows indicating the dying cells. PI uptake indicates the percentage of dead cells which is shown in P_2 population.

Figure 3: Morphological changes and viability after electroporation in Pan02. Following 24hours post treatment viability was analyzed through flow cytometry and morphology by cytospinning. Electroporation leads to transient morphological changes in pancreatic cancer cells including the presence of cytoplasmic vacuoles and leads to increased uptake of drugs with

correspondence to their viability. Red arrows indicate the dead cells and blue arrows indicating the dying cells. PI uptake indicates the percentage of dead cells which is shown in P_2 population.

Figure 4: PANC-1 cell recovery in response to drug therapy alone vs. combination therapy consisting of drug of interest and simultaneous electroporation. Cells treated with ECT-bleomycin recovered less than the cells treated with ECT-Cisplatin and ECT-Oxaloplatin. Each well shown is a representative image of at least nine similar wells (three independent experiments). Data also presented in bar graphs as mean IKK ± SD M of three independent experiments.

Figure 5: Pan02 cell recovery in response to drug therapy alone vs. combination therapy consisting of drug of interest and simultaneous electroporation. Cells treated with ECT - bleomycin recovered less than the cells treated with ECT-Cisplatin and ECT-Oxaloplatin. Each well shown is a representative image of at least nine similar wells (three independent experiments). Data also presented in bar graphs as mean IKK ± SD M of three independent experiments.

Figure 6: Differential expression of stemness factors Oct4/3, Nanog and Sox2 after Electrochemotherapy. Following 48 hours post treatment immunofluorescence of PANC-1 cells grown on cover slips was done by using anti-Oct3/4 (green), anti-Sox2 (green) and anti-Nanog (green) antibodies. DAPI staining (blue) indicates cell nuclei. The blue arrowhead indicates that the expression of Oct3/4 in untreated cells is comparatively higher compared with treated cells. The white arrowhead indicates a cell expressing higher quantity of Nanog stem cell marker.

Figure 7: Differential expression of stemness factors Oct4/3, Nanog and Sox2 after Electrochemotherapy. Following 48hours post treatment immunofluorescence of Pan02 cells grown on cover slips was done by using anti-Oct3/4 (green), anti-Sox2 (green) and anti-Nanog (green) antibodies. DAPI staining (blue) indicates cell nuclei. The blue arrowhead indicates that the expression of Oct3/4 in untreated cells is comparatively higher compared with treated cells. The white arrowhead indicates a cell expressing higher quantity of Nanog stem cell marker.

Figure 8: Expressional changes of stemness genes Oct4/3, Nanog and Sox2 post Electrochemotherapy. Total proteins were extracted (after 48hours growth) from human pancreatic cells line. Proteins were resolved by SDS polyacrylamide gel electrophoresis and analyzed by western blot. The antibodies used in the western blot analysis are indicated on the left. The relative change in expression of Nanog, Oct3/4 and Sox2 are graphically presented in (a), (b) and (c) respectively. Red box indicates enhanced expression of Nong after ECT, whilst blue box indicates relative decrease in expression after treatment. β -Actin was used as a loading control.